A098E Folliculogenesis, oogenesis, and superovulation

Bisphenol S affects *in vitro* early developmental oocyte competence in ewe

Alice Desmarchais, Ophélie Téteau, Pascal Papillier, Sébastien Elis

UMR 85 PRC, CNRS, IFCE, INRA, Université de Tours, 37380, Nouzilly, France.

Keywords*:* Bisphenol S, oocyte, ewe.

Bisphenol A (BPA), an estrogenomimetic endocrine disruptor, causes deleterious effects on oocyte meiosis and maturation (Machtinger, R. Reprod Biomed Online. 29(4): p. 404. 2014). BPA was banned from food industry and replaced by structural analogs including Bisphenol S (BPS). Some studies on fish and rodent species, reported BPS effects on reproduction similar to BPA effects (Uzumcu, M. Reprod Toxicol. 23(3): p. 337. 2007; Giulivo, M. Environ Res. 151: p. 251. 2016; Ullah, H. Chemosphere. 152: p. 383. 2016). This study aims, therefore, to assess the acute effects of low doses of BPS during *in vitro* maturation on oocyte developmental competence in ewe, as bisphenol resistance was previously reported in rodent species. Cumulus-oocyte complexes (COC) were collected from ovine follicles > 2 mm (n=3789 ovaries). First, COC underwent *in vitro* maturation (IVM) for 24h (Paramio, M.T. Theriogenology. 86(1): p. 152. 2016), in untreated condition (control) or in presence of BPS (Sigma, Saint Quentin Fallavier) at low doses (1 µM, 10 µM) and at environmental doses (1 nM, 10 nM, 100 nM) (Liao, C. Environ Sci Technol. 46(12): p. 6860. 2012). Oocyte viability was assessed with Live dead® staining (Thermofischer, Illkirch, France) and fluorescence microscope observation (Zeiss, Munich, Germany) (n= 1159 oocytes). Nuclear oocyte maturation rate was evaluated by metaphase II oocyte count after chromatin Hoechst staining (Sigma, Saint Quentin Fallavier, France) (n= 978 oocytes). Then, matured COC were *in vitro* fertilized (IVF) and developed (IVD) during 7 days in SOF medium (Zhu, J. Int J Vet Sci Med. 6(Suppl): p. S15. 2018). Cleavage and blastocyst rates were determined on day 2 and on day 7 post-IVF respectively, by microscope observation (n= 2280 oocytes). Data were analyzed using logistic regression and generalized linear model (R package Rcmdr, R version 3.5.3).

Our results showed that, BPS 1 µM and 10 µM do not affect oocyte viability rate (98% [n = 245] and 97.2 % [n = 282] respectively) compared to control (99 %, n = 289). Metaphase II oocyte rate is decreased by 13 % with BPS 10 µM (76,6%, p = 0.0008) compared to control (88%). Among fertilized COC (about 300 per condition), the very low dose BPS 1 nM significantly increased cleavage rate by 28.4 % (70.1%) compared to control (54.6%, p= 0.0003). On the contrary, BPS 1 µM decreased by 12.7 % the cleavage rate (47.6%) compared to control (p = 0.004). Among cleaved embryos, BPS 10 nM and BPS 1 µM decreased blastocyst rate respectively by 34.8% (14.2%, p = 0.046) and by 42.6 % (12.5%, p= 0.017), compared to control (21,8%). Particularly, BPS 1µM significantly reduced blastocyst hatching rate by 65 % (3.3%, p = 0.032) compared to control (9.4%). BPS during *in vitro* maturation negatively affects ovine cleavage and blastocyst rates. Our data suggest BPS negatively influences early developmental oocyte competence. Further studies are needed to investigate the potential BPS effect on estrogen receptors transcripts and on signaling pathways.

Supported by INRA, Region Val-de-Loire (project BEMOL) and the French National Research Agency (project ANR-18-CE34-0011-01 MAMBO). We thank A. Arnould and T. Delpuech for ovine ovary collection.

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Global transcriptome alterations in porcine oocytes with different developmental competence

Ahmed Gad[1,2], Lucie Nemcova[1], Matej Murin[1], Jiri Kanka[1], Jozef Laurincik[1,3], Radek Prochazka[1]

[1]Institute of Animal Physiology and Genetics of the Czech Academy of Sciences, Liběchov, Czech Republic; [2]Department of Animal Production, Faculty of Agriculture, Cairo University, Giza, Egypt; [3]Constantine the Philosopher University in Nitra, Nitra.

Keywords*:* oocyte, transcriptome, follicle size.

Although our knowledge regarding oocyte quality and development has improved significantly, molecular mechanisms regulating and determining oocyte developmental competence are still unclear. Therefore, the objective of this study was to identify and analyze the transcriptome profiles of porcine oocytes derived from different sized follicles and exhibited different developmental competence using RNA high throughput sequencing technology. Cumulus-oocyte complexes (COCs) of the same grades were aspirated from medium (MO; 3-6 mm) or small (SO; 1.5-1.9 mm) ovarian follicles and tested for developmental competence and chromatin configurations. After aspiration and removal of cumulus cells, oocytes were stained with Hoechst 33342 and chromatin configurations were assessed under a fluorescence microscope. COCs from the two groups were matured and cultured in vitro after parthenogenetic activation according to our previous protocol (Prochazka *et al.* 2011*, Reproduction* 141:425-435). After 144 h, the ability of embryos to reach the blastocyst stage was analyzed. For RNA sequencing, RNA libraries were constructed from both oocyte groups (three replicates each, n= 360) and then sequenced on an Illumina HiSeq4000. Raw expression data were normalized using the trimmed mean of M-values (TMM) normalization method. The differential expression analysis was done using the statistical Bioconductor software package EdgeR. Oocytes of MO group showed significantly higher blastocyst rate compared to the SO group (33.41±7.82 vs 15.51±3.44, respectively). MO group exhibited a significantly higher proportion of surrounded nucleolus chromatin configuration compared to the SO group which exhibited a higher percentage of the non-surrounded nucleolus configuration. Transcriptome analysis showed a total of 14,557 genes were commonly detected in both oocyte groups. A group of 930 genes was representing the top highly expressed genes (>5000 reads in each replicate) including genes related to cell cycle and oocyte meiosis and quality (*CCNB1, CCNB2, ESPL1, CPEB1, CUL1, CDC25B, CDC27, BMP15*, and *GDF9*). Differential expression (DE) analysis revealed 60 up- and 262 down-regulated genes (FDR< 0.05, FC≥ 1.5) in MO compared to SO group. *ACOD1, TNFSF11, and OAZ3* were among the top up-regulated genes, while *KCNJ14, IQCA1, CLDN15,* and *IGFBP2* were among the top down-regulated genes. Ontological classification of DE genes indicated that regulation of actin cytoskeleton, oxidative phosphorylation, and ECM-receptor interaction were among the significantly enriched pathways. In addition, biological processes related to cell growth and signaling, transcription, cytoskeleton, and extracellular matrix organization were among the highly enriched in DE genes. In conclusion, this study provides new insights into the transcriptome alterations of oocytes in relation to developmental competence.

This work was supported by projects CZ.02.1.01/0.0/0.0/15_003/0000460 from OP RDE, VEGA 1/0022/15, VEGA 1/0327/16 and APVV-14-0001.

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PGE2 concentration of the follicular fluid as a measure of heterogeneity of the response to hormonal stimulation of the bovine ovarian follicle

Christophe Richard[1], Gilles Charpigny[1], Olivier Dubois[1], Valérie Gelin[1,2], Fabienne Nuttinck[1]

[1]UMR BDR, INRA, ENVA, Université Paris Saclay, Jouy en Josas, France; [2]INRA, UCEA Bressonvilliers, 91630 Leudeville, France.

Keywords*:* ovarian-stimulation, PGE2, bovine.

The LH surge promotes prostaglandin E2 (PGE2) production within the preovulatory follicle. Oocyte microenvironment levels of PGE2 affect the developmental competence after fertilization. This study aimed to characterize the follicular fluid PGE2 enrichment during superovulation treatment. Six heifers (Holstein, 20.3 +/-0.85 months old) received FSH (Stimufol®, Reprobiol, Belgium): half dose, ie, 250 µg of porcine follitropin (pFSH), combined with 50 µg of porcine lutropin (pLH). At the rate of 8 injections, in degressive dose, spread over 4 days. PGF2α (Estrumate®, MSD Santé Animale, France) was injected at the same time as the 5th injection of Stimufol®.LH peak was assumed to occur between 35 and 40 hours after the PGF2α injection. Individual sampling of fluid from antral follicles was performed by ovum pick up 12 hours before PGF2α injection and 60 hours after PGF2α injection. This protocol was designed to allow the collection of fluids from ovarian follicle containing either a pre-matured or a matured oocyte. Each heifer was his own control as we took the "pre-matured" follicular fluid on a first ovary and the "matured" follicular fluid on the 2nd ovary, 3 days later. The punctures were repeated twice and were cross-checked for the next repetition to evaluate the impact of the ipsi or contralateral side of the corpus luteum (CL) on the follicular fluid composition. The volume of fluid was measured for each punctured follicle. The PGE2 concentration of the follicular fluid was measured by Elisa (Cayman Chemical) to determine the progress of terminal follicular differentiation. An average of 13 +/- 5.06 and 28 +/-13.9 follicles were punctured per session/heifer for respectively pre-matured (n=78) and matured (n=169) follicles. The mean collected volume differed between the two groups (pre-matured: 0.229+/-0.213 ml/follicle; matured: 0.575+/-0.379 ml/follicle; two samples t-test, pval<0.0001). No effect of the side of CL on fluid volume was detected (2-way Anova, p=0.397). The PGE2 concentration was determined in 25 pre-matured follicles and 127 matured follicles. The mean PGE2 concentration significantly differed between the two groups of follicular fluids (pre-matured: 7.2 +/-7.5 ng/ml; matured: 60.2 +/-58.6 ng/ml) No effect of the side of CL was detected (p=0.278). Surprisingly, there was no linear relationship between fluid volume and PGE2 concentration (adjusted R-squared: -0.0002, p-value=0.327). PGE2 concentrations were very spread out within the matured group. This important dispersion (Interquartile range=58.6 ng/ml) indicated that despite follicle growth in response to hormonal stimulation (FSH/LH) the ability of follicular granulosa and cumulus cells to synthesize PGE2 was imperfectly achieved. Only 48% of the follicular fluids in the mature group had higher PGE2 levels than those in the premature follicle group. In conclusion, despite the ability of the stimulation treatment to promote growth of many follicles, there was a great heterogeneity in terms of PGE2 synthesis. This alteration could represent defective signaling mechanisms that could impact the developmental competence of the oocyte.
Acknowledgements: APISGENE.

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Effects of bisphenol S on ovine primary granulosa cells *in vitro*

Ophélie Téteau, Manon Jaubert, Alice Desmarchais, Pascal Papillier, Sébastien Elis

INRA Centre Val de Loire, France.

Keywords*:* Bisphenol S, Granulosa Cells, Steroidogenesis.

Bisphenol A (BPA), a plasticizer used in food and drink packaging, medical devices and paper products (Giulivo M. et al., Environmental Research, Vol. 151: 251-264, 2016), has been prohibited in food industry due to its deleterious endocrine effects on both male and female reproduction (Bloom M.S. et al., Fertility and Sterility, Vol. 106: 857-863, 2016). Thus, BPA has been recently replaced by a structural analogue: bisphenol S (BPS). While its presence is exponentially increasing, BPS use is not regulated and its effects are still poorly understood, particularly on female reproduction. Several studies, especially in fish and rodents, already showed that BPS exhibits impacts similar as BPA in terms of both effects and intensity on the reproductive functions of these species, but this comparison BPA vs BPS was not yet study on granulosa cells (GCs) (Chen D. et al., Environmental Science & Technology, Vol. 50: 5438-5453, 2016 ; Rochester J.R. et al., Environmental Health Perspectives, Vol. 123: 643-650, 2015). GCs are essential for female reproductive function. They proliferate and secrete the hormones: progesterone and estradiol to allow the growth and maturation of the follicle and oocyte. The aim of this study is thus to investigate the *in vitro* effects of both BPS and BPA on ovine primary GCs. The ewe model was chosen as it is a relevant animal model for women reproduction. After follicle aspiration of approximately 1000 ovaries from local slaughterhouses, GCs were collected, purified and treated in complemented serum-free Mc Coy Medium, in absence (control) or presence of increasing concentration of BPS or BPA (1 nM, 10 nM, 100 nM, 1µM, 10 µM, 50 µM, 100 µM and 200 µM) for 48 hours. Progesterone and estradiol levels (12 and 5 independent cultures respectively) were measured by ELISA in the supernatant and normalized to the protein concentration of each well. Cell proliferation (13 independent cultures) was measured by ELISA assay after BrDU (BromoDesoxyUridine) incorporation. Data were analyzed using non-parametric permutational ANOVA and Tuckey post-hoc test. Our results showed that BPS did not affect cell proliferation, in contrast to BPA which significantly reduced cell proliferation at 50 µM ($P = 0{,}0007$) compared to the control. On the other hand, BPS significantly decreased progesterone secretion from 10 µM onwards (- 22 %; $P = 0{,}0038$) , whereas BPA lowered the level of progesterone only at 100 and 200 µM ($P < 0{,}0001$) compared to the control. BPS and BPA significantly increased estradiol secretion similarly from 10 µM onwards (+ 198 % $P = 0{,}0075$ vs. + 259 % $P < 0{,}0001$, respectively) compared to the control. These first results showed that BPS exhibits similar effects as BPA on steroidogenesus in ovine primary GCs, but not on cell proliferation. BPS even affected progesterone secretion at lower dose compared to BPA. Thus, BPS is probably not a safe alternative to BPA. Mechanisms disrupted by these molecules are currently studied in ovine primary GCs.

Supported by INRA, Region Val-de-Loire (project BEMOL) and the French National Research Agency (project ANR-18-CE34-0011-01 MAMBO).

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Effect of LIF, IL-6 and IL-11 on microRNA expression of bovine cumulus cells and oocytes matured *in vitro*

Meritxell Vendrell-Flotats[1,2], Leanne Stalker[3], Manel Lopez-Bejar[2], Marc Yeste[4], Teresa Mogas[1], Jonathan LaMarre[3]

[1]Dept. Medicina i Cirurgia Animals, Universitat Autonoma de Barcelona, Spain; [2]Dept. Anatomia i Sanitat Animals, Universitat Autonoma de Barcelona, Spain; [3]Dept. Of Biomedical Sciences, University of Guelph, Canada; [4]Dept. de Biologia, Universitat de Girona, Spain.

Keywords*:* oocyte, microRNA, cytokines.

Members of the interleukin (IL-6) family of cytokines including leukemia inhibitory factor (LIF), IL-6 and interleukin-11 (IL-11) are important for reproductive function, and the expression of specific cytokines is actually required for ovulation. They participate in follicular growth and development, oocyte maturation and implantation. IL-6 cytokines stimulate the intracellular Janus kinase/signal transducer, thereby activating JAK/STAT, MAP-kinase and PI(3)-kinase pathways. This alters downstream expression of genes and microRNAs (miRNAs) in oocytes and follicular cells, creating a microenvironment that improves oocyte quality and competency. However, the putative involvement of miRNAs in the JAK/STAT signal transduction pathway activated by members of the IL-6 family has not been fully elucidated. We, therefore, characterizedthe effects of LIF, IL-6 and IL-11 on miRNA expression in bovine cumulus-oocyte complexes matured*in vitro*. We assessed the expression of *miR-21*, *miR-155*, *miR-34c*and *miR-146a*, miRNAs previously implicated in oocyte maturation and cumulus expansion. Oocytes were distributed in 5 groups: GV (germinal vesicle), Control (matured in TCM199 + 10% FBS + FSH + LH + E2), LIF (TCM199 + 25 ng/mL LIF), IL-6 (TCM199 + 10 ng/mL IL-6), IL-11 (TCM199 + 5 ng/mL IL-11) and non-supplemented (TCM199). After 24h of IVM, cumulus cells were stripped from oocytes and both cumulus cells and oocytes were collected for miRNA extraction and qPCR analysis. The effects of treatment were analyzed by one-way ANOVA followed by a Sidak test ($p<0.05$). *MicroRNA-21*expression was significantly higher in cumulus cells from the control (FBS) and LIF groups and was higher in LIF-treated oocytes compared to TCM199 alone. IL-11 treatment increased *miR-146a*expression in oocytes while no significant differences were observed in the levels of *miR-146a*in cumulus cells. In cumulus cells,*miR-155*was significantly higher in controls, compared to oocytes, where no differences were observed between groups. The presence of cytokines during maturation had no effect on *miR-34c*expression in cumulus cells or oocytes in any group. miR-21 seems to be one of the most relevant miRNAs in oocyte function. It is the most abundant miRNA in cumulus cells in bovine. It is considered as an indicator of oocyte quality because it increases along oocyte maturation, when the oocyte becomes competent for fertilization. And also, for its anti-apoptotic role, as some of its target genes are related to apoptosis. miR-21 inhibition leads to an increase of active caspase 3 in granulosa cells, what results in an increased apoptosis. In conclusion, LIF addition to the maturation media may improve oocyte quality through increased expression of*miR-21*.It is relevant that LIF without serum and hormones could create a response in miR-21 similar to that in the controls. Further studies to evaluate the potential effects and mechanisms of action of LIF on bovine oocytes are warranted.
Supported by MCIU, Spain (AGL2016-79802-P) and by the OECD.